

ZINC, AN ESSENTIAL METAL ION
FOR BEEF LIVER δ -AMINOLEVULINATE DEHYDRATASE

Albert Cheh and J. B. Neilands

Department of Biochemistry
University of California
Berkeley, California 94720

Received October 25, 1973

Isolated beef liver δ -aminolevulinate dehydratase was found to contain variable levels of different metal ions. The addition of zinc ion restored activity to an apoenzyme prepared with the aid of chelating agents. It seems likely that zinc is the specific metal ion required by this enzyme.

Mammalian δ -aminolevulinate dehydratase (ALA-dehydratase) is severely inhibited by chelating agents of diverse structures (1,2) and the presence of various metal ions in the purified enzyme has been reported (2). We found that the enzyme from beef liver contained variable amounts of Zn, and traces of Cu and Fe, but never enough of any one of these to afford stoichiometry with the multiple subunits possessed by this relatively high molecular weight protein. It appears that the usual procedures for isolation yields a largely metal-free protein which, upon high dilution in the assay solution, acquires sufficient metal ion for apparent full activity. By assaying either the isolated enzyme or the apoenzyme under drastically suboptimal conditions, which required the use of much higher levels of protein, adventitious metal contamination was avoided (3) and activity could be titrated to a maximum value by addition of increments of zinc.

ALA-dehydratase was assayed by the method of Gibson *et al.* (4), using 20 mM mercaptoethanol as activator. The unit was defined as one millimicro-mole porphobilinogen per hour. Protein was determined on trichloroacetic acid (TCA) precipitates by the method of Lowry *et al.* (5). The enzyme was purified from frozen beef liver through the DEAE-Cellulose stage of Wilson

et al. (1), followed by affinity chromatography on a column of acylated amino-levulinic acid attached to Sepharose 4B through a hydrocarbon arm consisting of p-aminobenzoyl-6-aminohexanoic acid (6). Potassium levulinate, a competitive inhibitor, was used at 10 mM concentration to elute the enzyme. Metal analyses were performed with a Perkin Elmer Model 303 atomic absorption spectrophotometer. SDS gels were run by the method of Weber and Osborne (7). Precautions were taken to avoid contamination by adventitious metals (8,9). Apoenzyme was formed by dialysis against 0.01M EDTA, pH 7 for several hours at 4°. It was then passed through an 0.9 x 15 column of Bio-Gel P6 equilibrated with 0.05M tris-HCl, pH 7.2 (3). Assays to monitor titration of apoenzyme by zinc contained: 5 μ l of 1M Na-phosphate, pH 6.8, enzyme, H₂O, and metals up to a volume of 85 μ l. After 20 minutes at 37°, 5 μ l of 0.4M mercaptoethanol were added. After 30 minutes at 37°, the tubes were placed on ice and 10 μ l of 0.01M neutralized substrate were added. The reaction was stopped after 5-20 minutes by addition of 100 μ l of 0.1M HgCl₂-10% TCA. Following centrifugation 150 μ l aliquots were added to 150 μ l modified Ehrlich's reagent (10) and the O.D.₅₅₅ read after 10 minutes.

The yield of δ -ALA dehydratase, after 700-1000 fold purification, from 200g of liver was about 12% of material with specific activity of 13,000-15,000 units/mg. The enzyme was homogeneous by polyacrylamide gel electrophoresis and by ultracentrifugation analyses. The preparations were similar in purity to those obtained by using the method of Wilson et al. (1) carried through its entirety; the affinity column has the advantage of allowing final purification from the DEAE column in a few hours rather than several days. The method of Gurba et al. (2) which achieved a less than 200 fold purification appears to be inadequate for producing a pure enzyme.

The zinc content of the purified enzyme ranged from 0.5 to 2.0 atoms/280,000 grams of protein. Copper and iron ranged from 0.1 to 1.0 and 0.1 to 0.5 atoms, respectively. Manganese, cobalt, cadmium, and nickel were absent.

Concentration of column effluents by ammonium sulfate precipitation, rather than by ultrafiltration with Diaflo PM 10 membranes, tended to lower the zinc and raise the copper and iron content of the enzyme despite the use of "enzyme grade" ammonium sulfate. The loss of zinc from aldolase crystallized in ammonium sulfate has been noted (3).

As seen in Fig. 1 an apoenzyme which contained less than 10% of the zinc

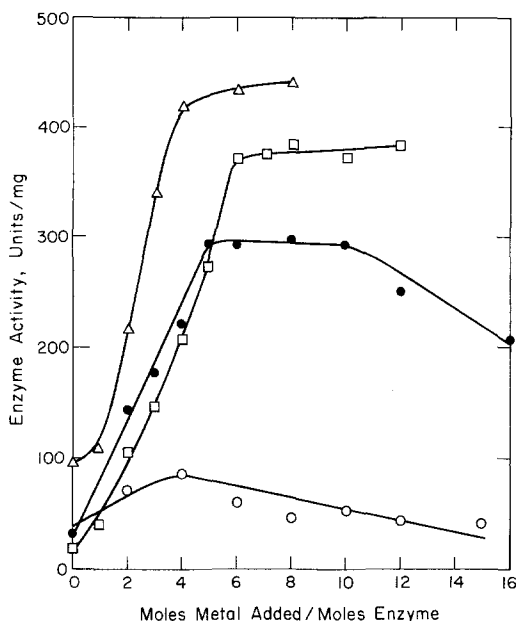


Figure 1. Titration of δ -aminolevulinate dehydratase with zinc and cobalt. Triangles-"native" enzyme $1 \times 10^{-6}M$ plus zinc, assayed in Na-phosphate, pH 6.8; squares-apoenzyme I, $2 \times 10^{-6}M$ plus zinc assayed in tris-HCl, pH 7.2; closed circles-apoenzyme II, $1.5 \times 10^{-6}M$ plus zinc, assayed in tris-HCl, pH 7.2; open circles-apoenzyme II, $1.5 \times 10^{-6}M$ plus cobalt, assayed in tris-HCl, pH 7.2.

initially present, showed very little activity in the lower temperature assay. Titration with between 5 and 6 gram atoms of zinc/280,000 grams protein led to maximum activity; excess zinc is inhibitory. A "native" enzyme containing 1.2 Zn/mole possessed 25% of the activity ultimately observed after saturation with zinc. These data demonstrate an absolute requirement for zinc by ALA-

dehydratase. Cobalt and manganese restored less than 20% of the activity while nickel, ferrous and ferric iron, cuprous and cupric copper, magnesium, calcium, and chromium III were inactive. Cadmium was the only metal ion, besides zinc, which restored full activity.

The subunit molecular weight was found to be 36,000, a figure which is in agreement with the data of Wilson *et al.* (1) but which is significantly lower than that reported by Gurba *et al.* (2). The molecular weight of the enzyme has been reported by different groups using various procedures as between 250,000 and 280,000 daltons (1,2). It thus appears that there are 7 to 8 subunits per mole. The 5 to 6 atoms of zinc required to reactivate the enzyme probably constitutes an upper limit because of kinetic and competition factors diminishing the effectiveness of the added metal ion. These data indicate that perhaps only half of the subunits are involved in zinc binding.

ALA dehydratase is a somewhat atypical metalloenzyme in that it loses much of its metal ion in the course of isolation.

Acknowledgments

The authors are indebted to Dr. John Gerhart for helpful suggestions. This work was supported in part by NIH grant No. AI-04156.

References

1. Wilson, E. L., P. E. Burger, and E. B. Dowdle, *Eur. J. Biochem.* 29, 563 (1972).
2. Gurba, P. E., R. E. Sennett, and R. D. Kobes, *Arch. Biochem. Biophys.* 150, 130 (1972).
3. Kobes, R. D., R. T. Simpson, B. L. Vallee, and W. J. Rutter, *Biochemistry* 8, 585 (1969).
4. Gibson, K. D., A. Neuberger, and J. J. Scott, *Biochem. J.* 61, 618 (1955).
5. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* 193, 265 (1951).
6. Cheh, A., Ph.D. thesis.
7. Weber, K. and M. Osborne, *J. Biol. Chem.* 244, 4406 (1969).
8. Himmelhoch, S. R., H. A. Sober, B. L. Vallee, E. A. Peterson, and K. Fuwa, *Biochemistry* 5, 2523 (1966).
9. Thiers, R. E., *Methods Biochem. Anal.* 5, 273 (1957).
10. Mauzerall, D. and S. Gramick, *J. Biol. Chem.* 219, 435 (1956).